Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/CA05/000211

International filing date:　　　　18 February 2005 (18.02.2005)

Document type:　　　Certified copy of priority document

Document details:　　Country/Office:　US
　　　　　　　　　　Number:　　　　60/545,732
　　　　　　　　　　Filing date:　　18 February 2004 (18.02.2004)

Date of receipt at the International Bureau:　　13 April 2005 (13.04.2005)

Remark:　　Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

PA 1287085

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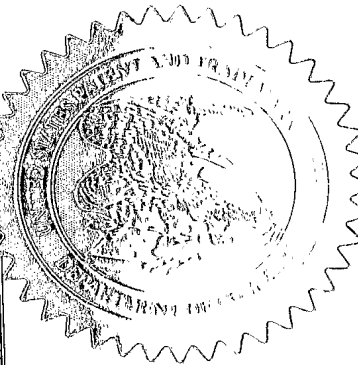
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET
This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

Express Mail Label No. EV 443839138 US

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☐ Additional inventors are being named on the separately numbered sheets attached hereto

TITLE OF THE INVENTION (280 characters max)

CpG-AMPLICON AND ARRAY PROTOCOL

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ENCLOSED APPLICATION PARTS (check all that apply)

X	Specification Number of pages	26	☐ CD(s), Number	[ ]
X	Drawing(s) Number of sheets	6	☐ Other (specify)	[ ]
☐	Application Data Sheet. See 37 CFR 1.76			

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,

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Docket Number: 04082-PPA

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Enclosed please find the following:

1. New U.S.A. provisional patent application entitled "CPG-AMPLICON AND ARRAY PROTOCOL", including specification and claims (26 pages), drawings (6 pages) Petronis et al, Inventors.

2. Form PTO/SB/16 duly executed.

3. Our check No. 01499, in the amount of $160.00, to cover the application filing fee.

4. Our post card. (Please date stamp and return.)

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If there are any additional fees required, please charge our Deposit Account No. 02-2839.

Thank you for your cooperation and assistance.

Respectfully submitted,

Robert M. Gamson
Reg. No. 32,986

RMG/jjr
Enclosures

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Draft Patent Application

CpG-Amplicon and Array Protocol

Applicant:

Centre for Addiction and Mental Health

Inventors:

Arturas Petronis
Axel Schumacher

February 18, 2004

- 1 -

CpG-Amplicon and Array Protocol

The present invention relates to methods and systems for epigenetic profiling. More specifically, the present invention relates to methods and systems for assessing methylation levels of nucleotide sequences.

BACKGROUND OF THE INVENTION

Many lines of evidence have shown that modification of cytosine bases residing in the dinucleotide sequence CpG in vertebrate genomes plays an essential role in regulating a variety of genome functions such as X chromosome inactivation, parental imprinting, inactivation of genomic retroelements, and differential gene expression. Across the human genome, about 80% of the CpG dinucleotides are heavily methylated, but some areas remain unmethylated, preferentially in the GC rich CpG islands [Bird, A.P., *CpG-rich islands and the function of DNA methylation. Nature, 1986. 321(6067): p. 209-13.*]. DNA methylation can perform its regulatory function through the differential marking of genes. Cytosine methylation is a stable but potentially reversable process which allows for the temporal and spatial-specific regulation of gene in higher organisms.

Several different strategies have been applied to detect methylated CpG dinucleotides in eukaryotic genomes (reviewed in [van Steensel, B. and S. Henikoff, *Epigenomic profiling using microarrays. Biotechniques, 2003. 35(2): p. 346-50, 352-4, 356-7*]). The most frequently used method is the bisulfite modification-based strategy, developed by Frommer et al. [Frommer, M., et al., *A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A, 1992. 89(5): p. 1827-31.*]. In this method, bisulfite converts unmethylated cytosine bases to uracil, whereas methylated cytosines remain unaltered. Such sequences can be directly sequenced using the Sanger sequencing method or can be interrogated using microarrays. In such microarrays, olinucleotide pairs that differ by having either a cytosine or a thymine at a methylatable position of a cytosine can discriminate the two nucleotides by incubating at a temperature that allows only exact matches between the probe and the olinucleotide Adorjan, P., et al.,

- 2 -

Tumour class prediction and discovery by microarray-based DNA methylation analysis. Nucleic Acids Res, 2002. 30(5): p. e21; Gitan, R.S., et al., *Methylation-specific oligonucleotide microarray: a new potential for high-throughput methylation analysis.* Genome Res, 2002. 12(1): p. 158-64; Balog, R.P., et al., *Parallel assessment of CpG methylation by two-color hybridization with oligonucleotide arrays.* Anal Biochem, 2002. 309(2): p. 301-10; Hou, P., et al., *A microarray to analyze methylation patterns of p16(Ink4a) gene 5'-CpG islands.* Clin Biochem, 2003. 36(3): p. 197-202.

Several other methods of providing methylation status on a global scale including microarray experiments have been published. In a method called differential methylation hybridization (DMH) [Huang, T.H., M.R. Perry, and D.E. Laux, *Methylation profiling of CpG islands in human breast cancer cells.* Hum Mol Genet, 1999. 8(3): p. 459-70.], genomic DNA (gDNA) from breast cancer cells were treated with the four-base cutter *MseI* that restricts gDNA into small fragments of 100-200 bp. This enzyme rarely cuts in CpG-rich regions, leaving many CpG islands intact. Differentially methylated CpG islands have been identified by ligation of end adaptors and subsequent PCR amplification.

Microarrays in this study contains DNA fragments representing various CpG islands. Several other publications used the step of enrichment for the hypermethylated fraction of a given genome [Yan, P.S., et al., *Applications of CpG Island microarrays for high-throughput analysis of DNA methylation.* J Nutr, 2002. 132(8 Suppl): p. 2430S-2434S; Yan, P.S., et al., *Use of CpG island microarrays to identify colorectal tumors with a high degree of concurrent methylation.* Methods, 2002. 27(2): p. 162-9; Shi, H., et al., *Triple analysis of the cancer epigenome: an integrated microarray system for assessing gene expression, DNA methylation, and histone acetylation.* Cancer Res, 2003. 63(9): p. 2164-71; Toyota, M., et al., *Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification.* Cancer Res, 1999. 59(10): p. 2307-12; Yan, P.S., et al., *Dissecting complex epigenetic alterations in breast cancer using CpG island microarrays.* Cancer Res, 2001. 61(23): p. 8375-80]. Amplification of non-methylated sequences is suppressed

- 3 -

by the digestion of the template DNA before PCR with the restriction enzymes *BstUI* and *HpaII*, which are blocked by methylation of their target sequence [Yan, P.S., et al., *Dissecting complex epigenetic alterations in breast cancer using CpG island microarrays*. Cancer Res, 2001. 61(23): p. 8375-80.]. The resulting hypermethylated DNA fraction was used to compare the methylation patterns from tumor and control tissues by hybridizing to microarrays containing randomly cloned genomic fragments that were enriched in CpG islands.

A related method uses a digestion step with *SmaI*, followed by digestion with *XmaI*, which is a methyl-insensitive isoschizomer of *SmaI* [Hatada, I., et al., *A microarray-based method for detecting methylated loci*. J Hum Genet, 2002. 47(8): p. 448-51.]. The cleavage with *SmaI* produces blunt end DNA fragment, whereas the cleavage products of *XmaI* contains protruding ends, which are ligated to specific *XmaI*-adaptors. After a PCR that uses primers specific for these adaptors, the resulting amplification products, which consist mainly of methylated 5'-CCCGGG-3' sequences, are hybridized to microarrays.

Another method that uses methylation-sensitive restriction enzymes for fractionating DNA was presented by Tompa et al. [Tompa, R., et al., *Genome-wide profiling of DNA methylation reveals transposon targets of CHROMOMETHYLASE3*. Curr Biol, 2002. 12(1): p. 65-8.]. This strategy used the methylation sensitive enzyme *MspI*, which cleaves 5'CCGG-3' but is blocked by methylation of the outer cytosine (^{m}5'-CCGG-3'). Digested DNA samples were size-fractionated on sucrose gradients (5%-30%) by ultracentrifugation as previously described [van Steensel, B., J. Delrow, and S. Henikoff, *Chromatin profiling using targeted DNA adenine methyltransferase*. Nat Genet, 2001. 27(3): p. 304-8.]. Gradient fraction containing plant DNA fragments smaller than 2.5 kb, as determined by gel-electrophoresis, were pooled and concentrated by isopropanol precipitation. Tester and control samples were then labeled with Cy3- or Cy5-dCTP by random priming and co-hybridized to microarrays that contained spotted PCR amplification products that primarily represented randomly chosen locations from the *Arabidopsis* genome [Tompa, R., et al., *Genome-wide profiling of DNA methylation reveals transposon targets of CHROMOMETHYLASE3*. Curr Biol, 2002. 12(1): p. 65-8].

- 4 -

There is a need in the art to develop new methods and systems for epigenetic profiling. Further there is a need in the art for new methods and systems for epigenetic profiling of chromosomes and genomes. Further still, there is a need in the art to develop methods and systems to assess methylation levels of probed loci such as repetitive elements, genes, imprinting elements, promoters, enhancer elements, intron sequences and whole genomes.

It is an object of the invention to overcome disadvantages of the prior art.

The above object is met by the combinations of features of the main claims, the sub-claims disclose further advantageous embodiments of the invention.

- 5 -

SUMMARY OF THE INVENTION

The present invention relates to methods and systems for epigenetic profiling. More specifically, the present invention relates to methods and systems for assessing methylation levels of nucleotide sequences.

According to the present invention there is provided a method of analysing the methylation state of one or more nucleotide sequences comprising the steps of:

a) selecting one or more genomic test nucleotide sequences from one or more subjects that exhibit a phenotype of interest and one or more corresponding genomic control sequences from one or more control subjects that lack the phenotype of interest;

b) separately digesting the genomic test nucleotide sequences and the genomic control sequences with one or more frequent cutting restriction endonucleases;

c) ligating adaptor nucleotide sequences to the ends produced from step b to produce ligated sequences;

d) cleaving the ligated sequences with one or more methylation sensitive restriction endonucleases to produce amplifiable test nucleotide sequences, non-amplifiable test nucleotide sequences, amplifiable control nucleotides sequences and non-amplifiable control nucleotide sequences;

e) amplifying the amplifiable test nucleotide sequences and amplifiable control nucleotide sequences to produce amplified test nucleotide sequences and amplified control nucleotide sequences;

f) labelling the amplified test nucleotide sequences from step e) and the non-amplified test nucleotide sequence from step d) with a first probe, and labelling the amplified control nucleotide sequence from step e) and the non-amplified control nucleotide sequence from step d) with a second probe;

g) hybridising the labelled products of step f) with an array comprising a series of nucleotide sequences that are capable of hybridising thereto;

h) determining the ratio of the signal emitted by the first probe relative to the ratio emitted by the second probe for each set of hybridised nucleotide sequences on the array.

- 6 -

The present invention also contemplates a method as defined above wherein the phenotype of interest comprises a disease, for example, but not limited to cancer, diabetes, Alzheimer's disease, schizophrenia or other disease. However, the present invention also contemplates employing the method of the present invention to analyze the methylation state or changes in the methylation state of one or more genomic nucleotide sequences in subjects, for example, but not limited to human subjects, or in cell cultures that are treated with a drug or the like, or that are subject to one or more specific physical stimuli or conditions.

The present invention further contemplates a method as defined above wherein the frequent cutting restriction endonuclease is selective for A/T rich sequences over C/G sequences, for example Csp6l, Tasl, or a combination thereof.

The present invention further contemplates a method as defined above wherein the probe is a chemically reactive fluorophore, for example, but not limited the first probe may be Cy3 and the second probe may be Cy5.

The present invention further contemplates a method of analysing the methylation state of one or more nucleotide sequences comprising the steps of:

a) selecting one or more genomic test nucleotide sequences from one or more subjects that exhibit a phenotype of interest, and one or more corresponding genomic control sequences from one or more control subjects that lack the phenotype of interest;

b) separately digesting the genomic test nucleotide sequences and the genomic control sequences with one or more methylation sensitive restriction endonucleases, to produce ends that can be ligated to an adaptor nucleotide sequence;

c) ligating adaptor nucleotide sequences to the ends produced from step b) to produce ligated sequences;

d) cleaving the ligated sequences with one or more CpG methylation specific endonucleases, to produce amplifiable test nucleotide sequences, non-amplifiable test nucleotide sequences, amplifiable control nucleotides sequences and non-amplifiable control nucleotide sequences;

- 7 -

e) amplifying the amplifiable test nucleotide sequences and amplifiable control nucleotide sequences to produce amplified test nucleotide sequences and amplified control nucleotide sequences;

f) labelling the amplified test nucleotide sequences from step e) and the non-amplified test nucleotide sequence from step d) with a first probe, and labelling the amplified control nucleotide sequence from step e) and the non-amplified control nucleotide sequence from step d) with a second probe;

g) hybridising the labelled products of step f) with an array comprising a series of nucleotide sequences that are capable of hybridising thereto;

h) determining the ratio of the signal emitted by the first probe relative to the ratio emitted by the second probe for each hybridised nucleotide sequence on the array.

The present invention further contemplates a method as defined above wherein the methylation restriction endonucleases comprise a cocktail comprising HpaII, Bsu15I (ClaI), Hin6I, AciI (SsiI) and TaiI.

Also contemplated by the present invention as defined above, CpG specific restriction endonuclease is McrBC.

The present invention further contemplates a kit comprising one or more genomic test nucleotide sequences, one or more corresponding genomic control nucleotide sequences, one or more frequent cutting restriction endonucleases, one or more specific adaptor nucleotide sequences, one or more methylation-sensitive restriction endonucleases, one or more CpG specific restriction endonucleases, one or more probes for labelling the nucleotide sequences, one or more microarrays capable hybridising to the genomic test and control nucleotide sequences, software for displaying and/or analysing the sequences hybridised to the microarray, reagents and/or enzymes for amplifying nucleotide sequences, or any combination thereof.

- 8 -

This summary of the invention does not necessarily describe all necessary features of the invention but that the invention may also reside in a sub-combination of the described features.

- 9 -

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

FIGURE 1 shows a diagrammatic depiction of an embodiment of the present invention.

FIGURE 2 shows a graphic depiction of a scatterplot of a comparison of a *McrBC* treated ligation versus an untreated ligation on the COMT-ARVCF array.

FIGURE 3 shows a typical 'smear' of DNA amplification products produced from assays at different temperatures.

FIGURE 4 shows a representative scatter plot of an experiment that detects methylation differences within repetitive elements (e.g. ALU or LINE elements) in different tissues. Grey circles indicate partially repetitive sequences (about 15 to about 30 copies/genome); while white circles indicate highly repetitive sequences (about >100 copies/genome).

FIGURE 5 shows a representative scatter plot of an experiment that detects methylation differences in the unique gene- and intergenic- sequences as well as of repetitive elements in the COMT-ARVCF chromosomal region on human chromosome 22.

FIGURE 6 provides of graphical depiction illustrating that methylation differences exist between DNA from human placenta tissue and DNA from post-mortem human striatum tissue.

- 10 -

DESCRIPTION OF PREFERRED EMBODIMENT

According to an embodiment of the present invention and referring generally to Figure 1, there is provided a method of analysing the methylation state of one or more nucleotide sequences. The method of the present invention may comprise the steps as shown on the left side of Figure 1, the right side of Figure 1, or both the left and right sides of Figure 1. In addition, the method of the present invention may comprise any combination of steps shown in Figure 1, for example on the right hand side, the left hand side or a combination thereof.

In an embodiment of the present invention, which is not meant to be limiting in any manner, the present invention provides a method of analysing the methylation state of one or more nucleotide sequences comprising the steps of:

a) selecting one or more genomic test nucleotide sequences from one or more subjects that exhibit a phenotype of interest, for example a disease such as but not limited to cancer, diabetes, alzheimers disease, schizophrenia or the like, and one or more corresponding genomic control sequences from one or more control subjects that lack the phenotype of interest;

b) separately digesting the genomic test nucleotide sequences and the genomic control sequences with one or more frequent cutting restriction endonucleases, preferably selective for A/T rich sequences, for example, but not limityed to Csp6I and TasI to produce ends that can be ligated to an adaptor nucleotide sequence;

c) ligating adaptor nucleotide sequences to the ends produced from step b to produce ligated sequences;

d) cleaving the ligated sequences with one or more methylation sensitive restriction endonucleases to produce amplifiable test nucleotide sequences, non-amplifiable test nucleotide sequences, amplifiable control nucleotides sequences and non-amplifiable control nucleotide sequences;

e) amplifying the amplifiable test nucleotide sequences and amplifiable control nucleotide sequences to produce amplified test nucleotide sequences and amplified control nucleotide sequences;

- 11 -

f) labelling the amplified test nucleotide sequences from step e) and the non-amplified test nucleotide sequence from step d) with a first probe, for example, but not limited to a chemically reactive fluorophore, for example, but not limited to the fluorophore being Cy 3, and labelling the amplified control nucleotide sequence from step e) and the non-amplified control nucleotide sequence from step d) with a second probe, for example, a chemically reactive fluorophore, for example, but not limited to the fluorophore being Cy 5;

g) hybridising the labelled products of step f) with an array comprising a series of nucleotide sequences that are capable of hybridising thereto;

h) determining the ratio of the signal emitted by the first probe relative to the ratio emitted by the second probe for each hybridised nucleotide sequences on the array.

The method as described above is shown diagrammatically by the left side of Figure 1.

In an alternate embodiment, there is provides a method of analysing the methylation state of one or more nucleotide sequences comprising the steps of:

a) selecting one or more genomic test nucleotide sequences from one or more subjects that exhibit a phenotype of interest, for example a disease such as but not limited to cancer, diabetes, alzheimers disease, schizophrenia or the like, and one or more corresponding genomic control sequences from one or more control subjects that lack the phenotype of interest;

b) separately digesting the genomic test nucleotide sequences and the genomic control sequences with one or more methylation sensitive restriction endonucleases, preferably a cocktail comprising HpaII, Bsu15I (ClaI), Hin6I, AciI (SsiI) and TaiI to produce ends that can be ligated to an adaptor nucleotide sequence;

c) ligating adaptor nucleotide sequences to the ends produced from step b to produce ligated sequences;

d) cleaving the ligated sequences with one or more CpG methylation specific endonucleases, for example, but not limited to McrBC to produce amplifiable test nucleotide sequences, non-amplifiable test nucleotide sequences, amplifiable control nucleotides sequences and non-amplifiable control nucleotide sequences;

- 12 -

e) amplifying the amplifiable test nucleotide sequences and amplifiable control nucleotide sequences to produce amplified test nucleotide sequences and amplified control nucleotide sequences;

f) labelling the amplified test nucleotide sequences from step e) and the non-amplified test nucleotide sequence from step d) with a first probe, for example, but not limited to a chemically reactive fluorophore, for example, but not limited to the fluorophore being Cy 3, and labelling the amplified control nucleotide sequence from step e) and the non-amplified control nucleotide sequence from step d) with a second probe, for example, a chemically reactive fluorophore, for example, but not limited to the fluorophore being Cy 5;

g) hybridising the labelled products of step f) with an array comprising a series of nucleotide sequences that are capable of hybridising thereto;

h) determining the ratio of the signal emitted by the first probe relative to the ratio emitted by the second probe for each hybridised nucleotide sequences on the array.

Further details of the method as described above may be found as depicted on the right side of Figure 1.

The present invention also contemplates a combination of the methods disclosed above, for example, but not limited to as shown generally by Figure 1.

The method of the present invention may be employed to identify specific nucleotide sequences that may be hypermethylated or hypomethylated in diseases relative to control genomic sequences and thus provide specific targets for therapeutic intervention. Further, the method may provide diagnostic and/or prognostic indicators for a disease.

The method of the present invention may also be employed with cell cultures, for example, but not limited to monitor and measure methylation changes after cells are treated with a biological agent, for example, but not limited to a drug, or after they are subjected to specific environmental conditions or stimuli.

- 13 -

The method of the present invention also may be used in combination with other methods to detect and quantify methylated DNA, for example, but not limited to the bisulfate method as described previously, or any other method as is known in the art.

The present invention further contemplates a kit comprising one or more genomic test nucleotide sequences, one or more corresponding genomic control nucleotide sequences, one or more frequent cutting restriction endonucleases, one or more specific adaptors nucleotide sequences, one or more methylation-sensitive restriction endonucleases, one or more CpG specific restriction endonucleases, one or more probes for labelling the nucleotide sequences, one or more microarrays capable hybridising to the genomic test and control nucleotide sequences, software for displaying and/or analysing the sequences hybridised to the microarray, reagents and/or enzymes for amplifying nucleotide sequences, or any combination thereof.

In an embodiment of the present invention, the method as described above provides for an array-based DNA methylation analysis of genomic nucleotide sequences, for example, but not limited to genes, repetitive elements such as but not limited to ALUs, LINEs etc.., enhancer elements, repressor elements, chromosomal regions, whole chromosomes/genomes or any compbination thereof. Representative steps of the method, that are not meant to be limiting in any manner are shown in Figure 1.

Referring now to Figure 1, there is shown a diagrammatic depiction of an embodiment of the present invention. The procedure is described for a "sample" and "control", however as will be evident to a person of skill in the art, the term "sample" may comprise a genomic test nucleotide sequence from a subject that exhibits a particular phenotype and the "control" may comprise a genomic control sequence from a control subject wherein the phenotype is absent. For example, but not wishing to be limiting in any manner, the sample may be from a subject that exhibits a disease phenotype, for example, but not limited to cancer (fro example but not limited to cancer of the breast, brain, bone, blood, prostate, skin cancer, etc) diabetes, alzheimer's, hypertension or any other disease. Conversely, the "control" does not exhibit the phenotype.

- 14 -

To enrich for the hypermethylated fraction of genomic DNA (see left side of Figure 1), the DNA is cleaved first with a frequent cutting restriction endonuclease, preferably a restriction endonuclease specific for A/T rich sequences, which produces ends in the DNA that can be ligated to an adaptor nucleotide sequence. Several enzymes with a 4-bp recognition sequence are known which produce sticky ends. For example, but not wishing to be limiting in any manner, *Csp6I* and *TasI* produce suitable ends. After the ligation of a *TasI* or *Csp6I* specific adaptor nucleotide sequences comprising internal sequences suitable for PCR amplification, the samples are cleaved with one or more methylation-sensitive restriction enzymes for example, but not limited to HpaII, AciI (SsiI), Bsu15I (ClaI) and/or Hin6I (HhaI), preferably a cocktail comprising 2 to about 5 or more of such enzymes. Consequently, substantially all unmethylated fragments are cut and cannot be amplified in the following PCR reaction. The PCR products of the sample and control are separately labeled with fluorescent dyes, combined, and hybridized to an oligo-array for example, but not limited to a COMT-ARVCF array, cDNA array or a CpG island microarray. The quantitation and analysis of array data permits a detailed comparison of the methylation status between sample and control.

To enrich the hypomethylated fraction of DNA in the sample and control, the DNA is cleaved with one or more, preferably a cocktail of methylation-sensitive restriction enzymes, for example, but not limited to *HpaII, Bsu15I (ClaI), Hin6I, AciI (SsiI), TaiI* or any combination thereof. Depending on the methylation status of the samples, these enzymes produce more or less fragments with sticky ends on which one or several adaptor nucleotide sequences can be ligated. Subsequently, the ligation products are subjected to an amplification procedure, which uses the adaptor sequences as primers. Therefore, as shown in Figure 1, depending on the enzymes chosen, it is possible to enrich hypo- or hypermethylated fragments of nucleotide sequences in a sample and contol. The resulting DNA fragments may be labeled in the PCR reaction (indirect labeling method) or labeled after the PCR reaction (direct labeling method). Finally, the labeled products are hybridized to arrays, which contain short oligo sequences, and the fluorescent markers are quantified and analyzed.

During the restriction cleavage of template DNAs, the reaction is preferably spiked with

- 15 -

array-specific oligonucleotides that function as normalization controls for example, but not limited to Lambda, Arabidopsis, prokaryotic plasmid sequences or a combination thereof.

In the embodiment shown on the right side of Figure 1, adaptor nucleotide sequences specific for the unmethylated CpG- dinucleotides are ligated to the hypomethylated DNA fragments whereas the hypermethylated (uncut) DNA regions remain unmodified. Long fragments, which could still contain methylated CpGs are cut by a CpG specific restriction endonuclease, for example, but not limited to McrBC. Without wishing to be considered limiting in any manner or bound by theory, McrBC is thought to cut only if two or more ^{m}CpGs are present in a DNA fragment. In a subsequent PCR reaction, primers complementary to the CpG-adaptors are used to preferentially amplify the hypomethylated DNA fragments in the sample and control.

Inset with Figure 1 is an example of a scatter plot derived from a "catechol-o-methyltransferase, armadillo repeat gene deleted in VCFS syndrome" (COMT-ARVCF) array, which reveals differences in DNA methylation patterns between samples and controls (see in particular the arrows in Figure 1).

In an embodiment of the present invention, the method employs specific adaptor nucleotide sequences that are highly specific for the protruding ends, generated by the aforementioned restriction enzymes. The adaptor nucleotide sequences preferably contain a small sequence-specific protruding end and a non-target homologous core sequence. The adaptor nucleotide sequences may also comprise a specific antisense-overhang that prevents tandem repeat formation and blunt-end ligation, a 'disruptor'' sequence, which disrupts the restriction sites after ligation, a non-5'-complementary end and a new restriction site that facilitates the cleavage of the adaptor from the target sequences if desired, or a combination thereof.

The following adaptor nucleotide sequences are exemplary and are not meant to limit the invention in any manner. The term "adaptor" and "adaptor nucleotide sequence" are used interchangeably.

- 16 -

Adaptor Nucleotide Sequences

(a) The CpG-overhang specific universal adaptor "U-CG1" for the hypomethylated DNA fraction is an adaptor that fits to DNA ends produced by the following methylation-sensitive restriction enzymes: *HpaII, MspI, Hin6I, Bsu15I (ClaI), AciI (SsiI), Psp1406I (AclI), Bsp119I (AsuII), HinII (AcyI), XmiI (AccI)* and the methylation-insensitive enzyme *TaqI*. The adaptor is the annealing product of the two primers:

U-CG1a: 5'-CGTGGAGACTGACTACCAGAT-3'

U-CG1b: 5'-AGTTACATCTGGTAGTCAGTCTCCA-3'

(b) The ACGT-overhang specific adaptor "ACGT-1" for the hypomethylated DNA fraction is an adaptor that fits to DNA ends produced by the methylation-sensitive restriction enzyme *TatI*. The adaptor is the annealing product of these two primers:

ACGT-1a: 5'-GAGACTGACTACCAGAT-3'

ACGT-1b: 5'-AGTTACATCTGGTAGTCAGTCTCACGT-3'

(c) The AATT-overhang specific adaptor "AATT-1" for the hypermethylated DNA fraction is an adaptor that fits to DNA ends produced by the methylation-insensitive restriction enzyme *TasI (TspEI)*. The adaptor is the annealing product of these two primers:

AATT-1a: 5'-GAGACTGACTACCAGAT-3'

AATT-1b: 5'-AGTTACATCTGGTAGTCAGTCTCAATT-3'

(d) The TA-overhang specific adaptor "TA-1" for the hypermethylated DNA fraction is an adaptor that fits to DNA ends produced by the methylation-insensitive restriction enzyme *Csp6I*. The adaptor is the annealing product of these two primers:

TA-1a: 5'-TATGAGACTGACTACCAGAT-3'

TA-1b: 5'-AGTTACATCTGGTAGTCAGTCTCA-3'

The adaptors are ligated by a T4 ligase to the restriction fragments produced by the enzymes specific for the hyper- and hypomethylated DNA fractions.

- 17 -

To enrich hyper- and hypomethylated fractions, both ligation-pools are subjected to
specific restriction cleavage prior to PCR amplification:

The hypomethylated ligation-fragments are cleaved by CpG specific restriction
endonuclease, for example, but not limited to, McrBC. McrBC from *Escherichia coli*
K-12 is a restriction enzyme that belongs to the family of AAA$^+$ proteins and cleaves
DNA containing methylcytosine on one or both strands [Sutherland, E., L. Coe, and
E.A. Raleigh, McrBC: a multisubunit GTP-dependent restriction endonuclease. J Mol
Biol, 1992. 225(2): p. 327-48; Kruger, T., C. Wild, and M. Noyer-Weidner, McrB: a
prokaryotic protein specifically recognizing DNA containing modified cytosine
residues. Embo J, 1995. 14(11): p. 2661-9; Stewart, F.J. and E.A. Raleigh,
Dependence of McrBC cleavage on distance between recognition elements. Biol
Chem, 1998. 379(4-5): p. 611-6.]. McrBC does not substantially cut unmethylated
DNA. Sites on the DNA recognized by McrBC may consist of two half-sites of the
form (G/A)^{m}C. Without wishing to be limiting in any manner or bound by theory,
these half-sites may be separated by up to about 3 kb, but are preferably separated by
about 55 to about 103 base pairs. McrBC acts upon a pair of Pu^{m}CG sequence
elements, thereby detecting a high proportion of methylated CpGs within the ligation-
fragments, but does not appreciably recognize HpaII/ MspI sites (CCGG) in which the
internal cytosine is methylated.

Referring now to Figure 2 there is shown a graphic depiction of a scatterplot of a
comparison of a McrBC treated ligation versus an untreated ligation on the COMT-
ARVCF array. As shown in Figure 2, McrBC treated fragments are cut and cannot be
amplified in the adaptor-PCR, therefore the signal will be much lower on the array
(shown in the Cy5 channel).

The hypermethylated ligation-fragments are preferably cleaved by specific combinations
of the restriction enzymes HpaII, MspI, Hin6I, Bsu15I (ClaI), AciI (SsiI), Psp1406I
(AclI), Bsp119I (AsuII), HinII (AcyI) or XmiI (AccI) depending on the stringency of the
approach. In an embodiment of the present invention, which is not meant to be limiting in
any manner, all of the enzymes are employed. In an alternate embodiment any of about 4
to about 9 enzymes may be employed. Also, it is contemplated that other enzymes not

- 18 -

listed below may be employed in combination with one or more enzymes listed above.

After restriction cleavage of the DNA-fractions, ligation products are amplified with primers specific for the adaptors used in the assay. Either the amplicon-fragments are labeled already during the PCR for example, but not limited to by allyl-labeling (the standard method uses aminoallyl (aa) nucleotide incorporation followed by coupling to N-hydroxysuccinimide (NHS) functionalized dyes (for example, but not limited to FluoroLink monofunctional dyes from Amersham/UK)) or a standard PCR with normal dNTPs is performed with subsequent labeling of the amplification products by random priming. For the amplification of small amounts of DNA (for example, but not limited to from micro-dissected tissues, the amplicons are amplified by a suitable enzyme for example, but not limited to the Phusion enzyme (MJ Research, Finland). Typically, a smear of DNA fragments is generated during the amplification reaction (see Figure 3).

Figure 3 shows a typical 'smear' of DNA amplification products. The annealing temperature influences the product size. Depending on the desired fragments-size, PCR conditions can be adjusted accordingly. Usually, an increased annealing/elongation temperature will lead to an increased product size. As larger PCR fragments can cross-hybridize to more of the oligos on the microarray, preferably they are avoided.

After labeling of sample and control samples with an appropriate probe, for example, but not limited to a fluorophore such as monofunctional Cy3/Cy5 dyes, the labeled samples may be hybridized to the microarray. In separate embodiments of the present invention, which are not meant to be limiting in any manner, the arrays may comprise human 1.7k cDNA arrays (UHN/Toronto, Can; which contain 1718 well characterized human ESTs), CpG island arrays (UHN/Toronto, Can), containing 12192 CpG island clones from the Sanger Centre/UK and custom made oligo-arrays for example, but not limited to an array spanning about 100 kb of the human COMT-ARVCF region on chromosome 22 have been successfully employed as arrays in practicing the method of the present invention. The present invention further contemplates the use of any array known in the art in the method of the present invention.

- 19 -

Design of the oligo-arrays

Without wishing to be limiting in any manner, epigenetic oligo-microarrays may be prepared on CMT-GAPS slides (Corning Inc.) or equivalent pre-processed microarray slides. Oligos for a desired chromosomal region (for example, but not limited to human LINE repetitive elements are preferably about 25bp to about 50 bp in length or longer. The sequence of the oligos is preferably derived from loci between methylation-sensitive restriction sites used in the method (more preferably *Acil*, *Hpall* and *Hin6l*). The oligos are preferably designed either between each adjacent restriction sites or for every second site, depending on the specificity desired for each chromosomal region.

The method of the present invention may be employed in a wide variety of applications, for example, but not limited to the detection of methylation differences within human repetitive elements (Figure 4).

Figure 4 shows a representative scatter plot of an experiment that detects methylation differences within repetitive elements (e.g. ALU or LINE elements) in different tissues. Grey circles indicate partially repetitive sequences (about 15 to about 30 copies/genome); while white circles indicate highly repetitive sequences (about >100 copies/genome).

In addition to the detection of repetative signals, the method may also be employed to detect methylation differences in unique gene-sequences as exemplified for the analysis of brain-tumors compared to control brains (see Figure 5).

Figure 5 shows a scatter plot of an experiment that detects methylation differences in the unique gene- and intergenic- sequences as well as of repetitive elements in the COMT-ARVCF chromosomal region on human chromosome 22. In this comparison the analysis of the oligo-arrays revealed a relative hypermethylation of repetitive elements in the brain tumor.

- 20 -

Referring now to Figure 6 there is shown an example wherein the method may be employed compare different tissues in respect to their methylation profile on chromosome 22. As shown in Figure 6, methylation differences exist between DNA from human placenta tissue and DNA from post-mortem human striatum tissue. The method reveals a significant temporal- and spatial methylation difference between these two tissue types. The further the location of a dot from the regression line, the larger the DNA methylation difference in the given location of the DNA fragment.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

Examples

Example 1: Profiling of epiG DRD2 using microarrays

In an embodiment of the present invention, the method may be employed to profile epiG DRD2 using microarrays. In the embodiment, a microarray is designed that is specific for epiG profiling of the full length of DRD2, including the very long (~250kb) intron 1. Without wishing to be limiting in any manner, the general principle of the 'epiG' array comprises the hybridization of the hypomethylated (or hypermethylated) fraction of genomic DNA (or the DNA fraction associated with acetylated, methylated, for example, but not limited to histones) to the microarray containing oligonucleotides that represent the genomic region of interest. Intensity of hybridization correlates with the DNA methylation status at the genomic locus

- 21 -

homologous to a specific oligonucleotide on the array. The microarray -based epiG
analysis of DRD2 comprises the following steps:

i)   Oligonucleotides for microarrays. Using the publicly available human genome
sequence of DRD2 plus wide upstream and downstream regions
(http://genome.ucsc.edu/). 40-50 base oligonucleotides (with aminomodifiers at the 5'
end) that cover the testable genomic region of ~350kb are designed. In epiG studies,
sufficient coverage is achieved by about 3-5 (or more) oligonucleotides per kilobase
of genomic DNA. Repetitive DNA elements may be excluded using the
RepeatMasker, which reduces the length of the target sequence from about 350kb to
about 200kb. This requires about 800 oligonucleotides that will be synthesized for
example, but not limited to at Qiagen, and then spotted on the glass at a specific
location, for example, but not limited to the UHN Microarray Facility.

ii)   DNA samples are extracted from the D2 expressing cell lines treated with i)
haloperidol; ii) clozapine; iii) haloperidol + VPA; iv) clozapine + VPA; v) VPA only,
and the control DNA is extracted from the identical cell line of the same age, but
without exposure to an antipsychotic. Two D2 receptor expressing cell lines are used:
HTB-18 (Y-79)57 (available from ATCC), and hNT58 (available from Layton
BioScience, Inc.).

iii)   Time intervals. DNA samples are extracted from each of the above treatments
after 1, 6, and 24 hours, and then 3, and 7 days (time intervals selected arbitrarily).

iv)   Preparation of the hypomethylated fraction of genomic DNA. Without wishing
to be bound by theory, a cocktail of methylation sensitive restriction enzymes, such as
Hpafl, Hin6I, AciI, TaiI, and a recent addition of McrBC, may interrogate 25%-50%
of all CpGs (Schumacher, Petronis et al; in preparation). In order to enrich the
hypomethylated fraction of genomic DNA, after digestion with DNA methylation
sensitive restriction enzymes, DNA adaptors are ligated to the restriction fragments,
which is followed by subsequent polymerase chain reaction (PCR) amplification using
primers that are complementary to the adaptors. PCR conditions are adjusted in such a

- 22 -

way that only fragments that are less than 1kb (i.e. short, digested, and therefore unmethylated) will amplify preferentially. The hypomethylated fraction of genomic DNA from matching pairs are then labeled with Cy3- (e.g., DNA from cells treated with haloperidol) and Cy5- (e.g., DNA from the control cells) and co-hybridized to the microarray. Each comparison is performed in duplicate or greater, and averaged intensities are used for the further analyses.

v)   Hybridizations are performed using standard array protocols as described herein, and scanning of microarrays may be perfromed at the UHN Microarray Facility using the GenePix software (Pro 3.1). The software gives a raw data output, which is normalized by NormalizingSuite 2.0 and subjected to further analysis using the home-made Excel Macros. A set of experiments on a gene using a microarray of 100+ oligos (more recently with 300+ oligos), shows consistent results of DNA methylation profiles of this region.

vi)   Data analysis. The analysis of hybridization profiles and identification of the drug induced epiG changes is straightforward. The hypomethylated fraction of DNA from treated cell lines is compared to the one from an untreated control, and scatter plot diagrams for each comparison will be generated. Hybridization signals that deviate from the regression line are sought.

The method of enriching hyper- and hypomethylated DNA fractions is different and improved compared to previously published methods. The method as disclosed herein is the first that uses a novel strategy for the enrichment of hypomethylated and hypermethylated fraction of genomic DNA, that efficiently compares the methylation status of CpG dinucleotides in test and control samples across large and very large segments of genomic DNA. In addition, the method employs an informative combination

- 23 -

of methylation sensitive restriction enzymes that cleave or do not cleave DNA containing methyl-cytosine on one or both strands and permits for a more stringent and detailed analysis of methylation profiles compared to the other methods known in the art.

The present invention also allows the analysis of very small tissue samples (e.g. from laser micro-dissected samples). The necessary amount of genomic DNA (gDNA) for one analysis may be as low as 50 pg (< 10 cells).

The array-based method as described above has also several advantages compared to the bisulfite-dependent methods. The methods that rely on the bisulfite method are commonly used but require labor-intensive and time-consuming cloning and sequencing steps, which can be skipped when using the method of the present invention. Further, the bisulfite-based strategies provide only information about specific residues that have been chosen in advance as being informative, whereas the method as described herein may be used to screen complete genomes for methylation differences. Moreover, if the bisulfite approach is used in the microarray format, the technique requires numerous permutations of oligonucleotides, which dramatically increases the costs for oligonucleotides or is limited to a relatively short DNA segment.

All citations are herein incorporated by reference.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

–24–

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method of analysing the methylation state of one or more nucleotide sequences comprising the steps of:

a) selecting one or more genomic test nucleotide sequences from one or more subjects that exhibit a phenotype of interest and one or more corresponding genomic control sequences from one or more control subjects that lack the phenotype of interest;

b) separately digesting the genomic test nucleotide sequences and the genomic control sequences with one or more frequent cutting restriction endonucleases;

c) ligating adaptor nucleotide sequences to the ends produced from step b to produce ligated sequences;

d) cleaving the ligated sequences with one or more methylation sensitive restriction endonucleases to produce amplifiable test nucleotide sequences, non-amplifiable test nucleotide sequences, amplifiable control nucleotides sequences and non-amplifiable control nucleotide sequences;

e) amplifying the amplifiable test nucleotide sequences and amplifiable control nucleotide sequences to produce amplified test nucleotide sequences and amplified control nucleotide sequences;

f) labelling the amplified test nucleotide sequences from step e) and the non-amplified test nucleotide sequence from step d) with a first probe, and labelling the amplified control nucleotide sequence from step e) and the non-amplified control nucleotide sequence from step d) with a second probe;

g) hybridising the labelled products of step f) with an array comprising a series of nucleotide sequences that are capable of hybridising thereto;

h) determining the ratio of the signal emitted by the first probe relative to the ratio emitted by the second probe for each set of hybridised nucleotide sequence on the array.

2) The method of claim 1, wherein said phenotype of interest comprises a disease such as cancer, diabetes, alzheimers disease, or schizophrenia.

3) The method of claim 1, wherein said frequent cutting restriction endonuclease is selective for A/T rich sequences over C/G sequences.

- 25 -

4) The method of claim 3, wherein said frequent cutting restriction endonuclease comprises Csp6I, TasI, or a combination thereof.

5) The method of claim 1, wherein said probe is a chemically reactive fluorophore.

6) The method of claim 5, wherein said flurophore is Cy 3 or Cy 5.

7) A method of analysing the methylation state of one or more nucleotide sequences comprising the steps of:

a) selecting one or more genomic test nucleotide sequences from one or more subjects that exhibit a phenotype of interest, and one or more corresponding genomic control sequences from one or more control subjects that lack the phenotype of interest;

b) separately digesting the genomic test nucleotide sequences and the genomic control sequences with one or more methylation sensitive restriction endonucleases, to produce ends that can be ligated to an adaptor nucleotide sequence;

c) ligating adaptor nucleotide sequences to the ends produced from step b) to produce ligated sequences;

d) cleaving the ligated sequences with one or more CpG methylation specific endonucleases, to produce amplifiable test nucleotide sequences, non-amplifiable test nucleotide sequences, amplifiable control nucleotides sequences and non-amplifiable control nucleotide sequences;

e) amplifying the amplifiable test nucleotide sequences and amplifiable control nucleotide sequences to produce amplified test nucleotide sequences and amplified control nucleotide sequences;

f) labelling the amplified test nucleotide sequences from step e) and the non-amplified test nucleotide sequence from step d) with a first probe, and labelling the amplified control nucleotide sequence from step e) and the non-amplified control nucleotide sequence from step d) with a second probe;

g) hybridising the labelled products of step f) with an array comprising a series of nucleotide sequences that are capable of hybridising thereto;

- 26 -

h) determining the ratio of the signal emitted by the first probe relative to the ratio emitted by the second probe for each hybridised nucleotide sequence on the array.

8) The method of claim 7, wherein said methylation restriction endonucleases comprise a cocktail comprising HpaII, Bsu15I (ClaI), Hin6I, AciI (SsiI) and TaiI.

9) The method of claim 7, wherein said CpG specific endonuclease is McrBC.

10) A kit comprising one or more genomic test nucleotide sequences, one or more corresponding genomic control nucleotide sequences, one or more frequent cutting restriction endonucleases, one or more specific adaptor nucleotide sequences, one or more methylation-sensitive restriction endonucleases, one or more CpG specific restriction endonucleases, one or more probes for labelling the nucleotide sequences, one or more microarrays capable hybridising to the genomic test and control nucleotide sequences, software for displaying and/or analysing the sequences hybridised to the microarray, reagents and/or enzymes for amplifying nucleotide sequences, or any combination thereof.

ABSTRACT OF THE DISCLOSURE

The invention can be summarized as follows. There is provided a method for amplifying hypermethylated genomic nucleotide sequences and/or hypomethylated genomic nucleotide sequences and comparing the methylation state between different samples, for example control and test samples. Also disclosed is a microarray based method for analysing hyper and/or hypomethylated genomic nucleotide sequence. Further, kits comprising reagents for practising the method are provided.
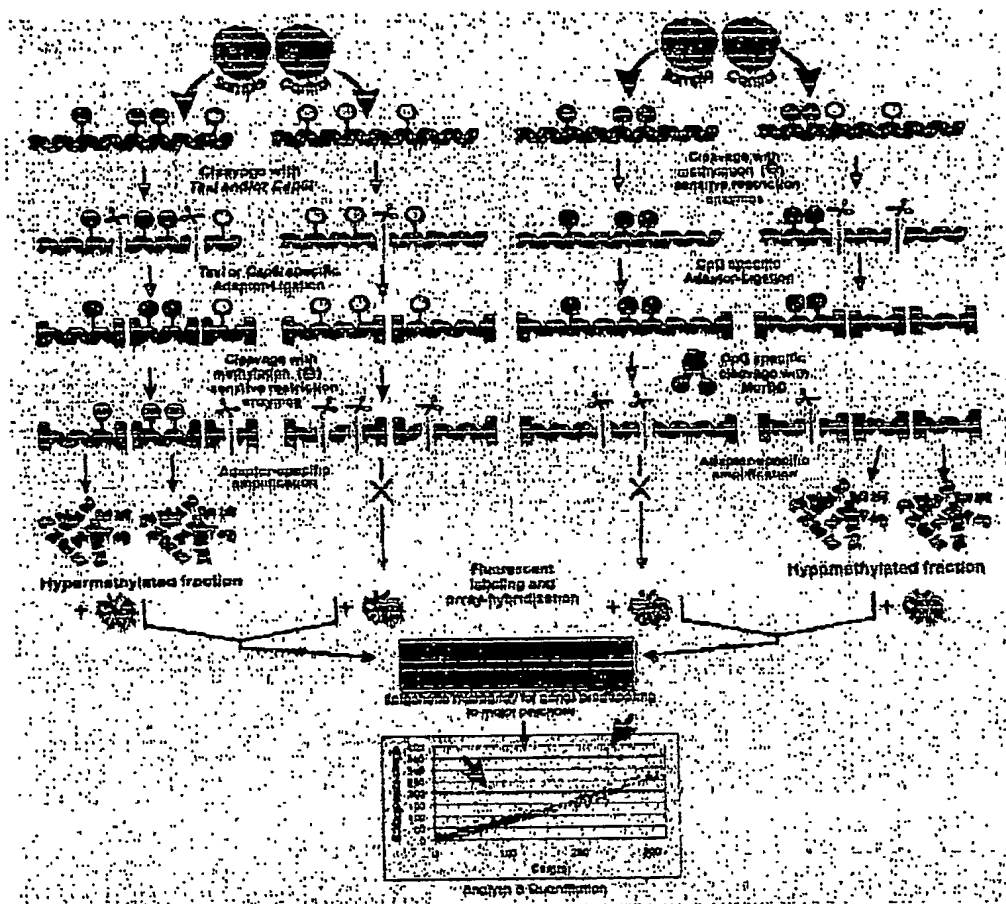
- 27 -

FIGURE 1

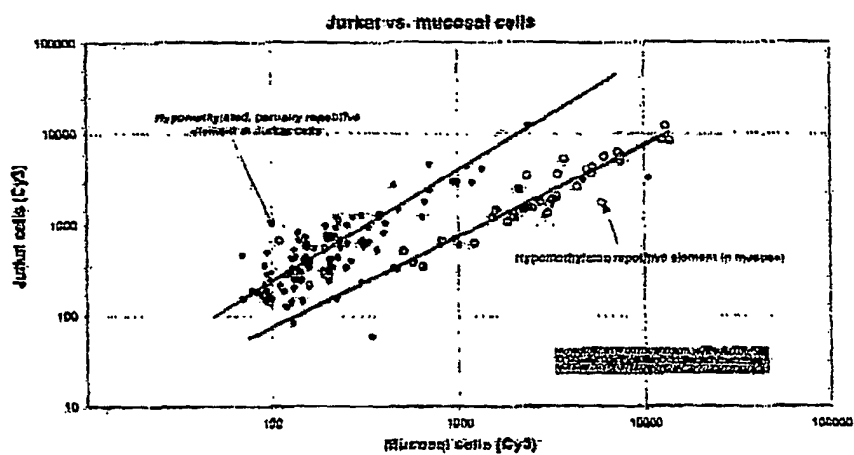- 28 -

FIGURE 2

FIGURE 3.

- 30 -

FIGURE 4

FIGURE 5

-32-

FIGURE 6